Supporting Information:

Ground state depletion microscopy: detection sensitivity of single-molecule optical absorption at room temperature

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Derivation of Equation 1

Considering only the ground state and the first excited state of a chromophore, the power attenuation by the single molecule under a tightly focused laser beam is the product of the power that hits the molecular absorption cross-section and the probability that the molecule stays in the ground state.

Assuming the excitation field in the beam waist is uniform, the power that hits the molecular absorption cross-section is $(\sigma/S)\cdot P$, where σ is the absorption cross-section for a single chromophore, S is the beam waist area, and P is the incident power. The probability that the molecule stays in the ground state is determined by the rate of excitation and relaxation. The rate of excitation,

$$k_{exc} = \frac{(\sigma/S)\cdot P}{h\nu}, \tag{S1}$$

where $h\nu$ is the photon energy. And the rate of relaxation,

$$k_{relax} = \tau^{-1}, \tag{S2}$$

where τ is the excited state lifetime of the molecule. Considering a time scale that is much longer than a photocycle, the transition between ground state and the excited state reaches a steady state, and the probability for the molecule to be at ground state is

$$p_{ground} = \frac{k_{relax}}{k_{relax}+k_{exc}} = \frac{k_{relax}}{k_{relax}+(\sigma/h\nu S)\cdot P}, \tag{S3}$$

Therefore the power attenuation is,

$$\Delta P = p_{ground}(\sigma/S)\cdot P = \frac{k_{relax}\cdot(\sigma/S)\cdot P}{k_{relax}+(\sigma/h\nu S)\cdot P}, \tag{S4}$$

which is Eq. 1.

Derivation of Equation 2

Assume that the incident power levels of pump beam and probe beam are P_{pump} and P_{probe}, respectively. In the ground state depletion microscopy, while the intensity of pump beam ν_1 is modulated, the probe beam ν_2 is always present interacting with the molecule. When the pump beam ν_1 is blocked, the probe beam ν_2 is absorbed by certain amount,

$$\Delta P_{off} = \frac{k_{relax} \cdot (\sigma / S) \cdot P_{probe}}{k_{relax} + (\sigma / h\nu S) \cdot P_{probe}} \quad \text{(S5)}$$

In contrast, when both ν_1 and ν_2 are exciting the molecule, the attenuation at the probe beam wavelength ν_2 will be

$$\Delta P_{on} = \frac{k_{relax} \cdot (\sigma / S) \cdot P_{probe}}{k_{relax} + (\sigma / h\nu S) \cdot (2P_{pump} + P_{probe})} \quad \text{(S6)}$$

Note that here we assume the difference in σ and $h\nu$ is negligible for ν_1 and ν_2. There is a factor of 2 in front of P_{pump} because the incident power of the pump beam when it is modulated on- is twice that of the average, assuming 100% modulation depth for the pump beam.

It is evident that, because the ground state population is depleted more when both ν_1 and ν_2 are present, the corresponding attenuation of the probe beam will be less compared to the case where the pump beam is blocked

$$\Delta P_{off} > \Delta P_{on} \quad \text{(S7)}$$

Therefore, the switching between pump beam on- and pump beam off- will create a modulation depth, $\delta\delta P_{probe}$,

$$\delta\delta P_{probe} \equiv \Delta P_{off} - \Delta P_{on} = \frac{2k_{relax} \cdot (\sigma^2 / S^2 h\nu) \cdot P_{pump} P_{probe}}{\left(k_{relax} + (\sigma / h\nu S) \cdot P_{probe}\right) \cdot \left(k_{relax} + (\sigma / h\nu S) \cdot (2P_{pump} + P_{probe})\right)}, \quad \text{(S8)}$$

which is Eq. 2.

Estimate of the signal of a single gold nanoparticle with ground state depletion microscopy

The absorption cross section of a 20 nm gold nanoparticle at its plasmon resonance is ~4×10^{-12} cm^2.[S1] It has been shown that after excitation by subpicosecond laser pulses, the kinetics of the recovering plasmon band is biexponential.[S2] The bleached ground state A recovers through a fast electron-phonon relaxation mechanism (1~4 ps),[S2, S3] and a slow phonon-phonon relaxation mechanism (~100 ps).[S3]

$$B \xrightarrow{k_1} C \xrightarrow{k_2} A$$

The rate constants for the fast relaxation (B to C) and the slow relaxation (C to A) are k_1 and k_2 respectively. We first calculate the temporal behavior of the absorption at the plasmon band by the gold nanoparticle after excitation by an ultrafast laser pulse.

Let states A and C have absorption cross sections σ_A and σ_C respectively, and assume that the absorption of B is negligible. Let p_A, p_B and p_C represent the probabilities that the gold nanoparticle is in state A, B, and C, respectively. The absorbance of the nanoparticle,

$$\text{Abs} \propto p_A\sigma_A + p_C\sigma_C \quad \text{(S9)}$$

At time zero,

$$p_{B,t=0} = 1 \quad \text{(S10)}$$

In the infinite time limit,

$$p_{A,t=\infty} = 1 \quad \text{(S11)}$$

At arbitrary time t, we have

$$p_A + p_B + p_C = 1 \quad \text{(S12)}$$

$$\frac{dp_C}{dt} = k_1 p_B - k_2 p_C \quad \text{(S13)}$$

$$\frac{dp_A}{dt} = k_2 p_C \qquad \text{(S14)}$$

The system of differential equations (Eq.S10 to S14) can be solved as

$$p_C = \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \qquad \text{(S15)}$$

$$p_A = 1 - \frac{k_2}{k_2 - k_1} e^{-k_1 t} - \frac{k_1}{k_1 - k_2} e^{-k_2 t} \qquad \text{(S16)}$$

Substitute Eq.S15 and Eq.S16 into Eq.S9, the absorbance of the nanoparticle can be rearranged as

$$\text{Abs} \propto \sigma_A + \frac{k_2\sigma_A - k_1\sigma_C}{k_1 - k_2} e^{-k_1 t} + \frac{k_1(\sigma_C - \sigma_A)}{k_1 - k_2} e^{-k_2 t} \qquad \text{(S17)}$$

Note that $k_1 \square\ k_2$. When $\sigma_C < \sigma_A$ and $\frac{k_2}{k_1} < \frac{\sigma_C}{\sigma_A}$, the absorbance as a function of time is biexponential, consistent with the measured kinetics of plasmon band recovery.[S2]

According to Eq.S17, the amplitudes of the fast and slow components in the biexponential kinetics of the plasmon band recovery are proportional to $\frac{k_2\sigma_A - k_1\sigma_C}{k_1 - k_2}$ and $\frac{k_1(\sigma_C - \sigma_A)}{k_1 - k_2}$, respectively.

The ratio of the amplitudes of the fast component and slow component was reported to be 1.5 using 380nm excitation light, and 9 using 600nm excitation light.[S2] We assume the ratio as 9 for the excitation wavelength that we use (532nm and 520nm).

$$\frac{\frac{k_2\sigma_A - k_1\sigma_C}{k_1 - k_2}}{\frac{k_1(\sigma_C - \sigma_A)}{k_1 - k_2}} = \frac{k_2\sigma_A - k_1\sigma_C}{k_1(\sigma_C - \sigma_A)} = 9 \qquad \text{(S18)}$$

According to refernece S3, $k_1 \approx \frac{1}{4\times10^{-12}\,s} = 2.5\times10^{11}\,s^{-1}$ and $k_2 \approx \frac{1}{100\times10^{-12}\,s} = 10^{10}\,s^{-1}$, therefore we have $\sigma_C = 0.9\sigma_A$.

We next calculate the power of the incident laser beam that is attenuated by a gold nanoparticle in a one beam CW experiment with the following kinetic scheme:

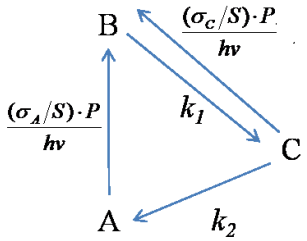

Figure S1. Kinetic scheme for contineous wave optical excitation and excited state relaxation of a gold nanoparticle.

Under continuous excitation, the probabilities of the three states of the gold nanoparticle reach a steady state. Therefore we have,

$$p_A + p_B + p_C = 1 \tag{S19}$$

$$\frac{dp_A}{dt} = k_2 p_C - \frac{(\sigma_A / S)P}{h\nu} p_A = 0 \tag{S20}$$

$$\frac{dp_B}{dt} = \frac{(\sigma_A / S)P}{h\nu} p_A - k_1 p_B + \frac{(\sigma_C / S)P}{h\nu} p_C = 0 \tag{S21}$$

This system of the equations (Eq.S19 to Eq.S21) can be solved as

$$p_A = \frac{1}{1 + \frac{(\sigma_A / S)P}{k_1 h\nu} + \frac{(\sigma_A / S)P}{k_2 h\nu} + \frac{(\sigma_A / S)(\sigma_C / S)P^2}{k_1 k_2 (h\nu)^2}} \tag{S22}$$

$$p_B = \frac{\frac{(\sigma_A / S)P}{k_1 h\nu} + \frac{(\sigma_A / S)(\sigma_C / S)P^2}{k_1 k_2 (h\nu)^2}}{1 + \frac{(\sigma_A / S)P}{k_1 h\nu} + \frac{(\sigma_A / S)P}{k_2 h\nu} + \frac{(\sigma_A / S)(\sigma_C / S)P^2}{k_1 k_2 (h\nu)^2}} \tag{S23}$$

$$p_C = \frac{\frac{(\sigma_A / S)P}{k_2 h\nu}}{1 + \frac{(\sigma_A / S)P}{k_1 h\nu} + \frac{(\sigma_A / S)P}{k_2 h\nu} + \frac{(\sigma_A / S)(\sigma_C / S)P^2}{k_1 k_2 (h\nu)^2}} \tag{S24}$$

The combined power attenuation from states A and C can be written as:

$$\Delta P = p_A(\sigma_A / S)\cdot P + p_C(\sigma_C / S)\cdot P = \frac{(\sigma_A + \frac{\sigma_C(\sigma_A / S)P}{k_2 h\nu})\cdot P / S}{1 + \frac{(\sigma_A / S)P}{k_1 h\nu} + \frac{(\sigma_A / S)P}{k_2 h\nu} + \frac{(\sigma_A / S)(\sigma_C / S)P^2}{k_1 k_2 (h\nu)^2}} \tag{S25}$$

By substituting $\sigma_C = 0.9\sigma_A$ into Eq.S25, we get

$$\Delta P = \frac{(\sigma_A + \frac{0.9\sigma_A(\sigma_A / S)P}{k_2 h\nu})\cdot P / S}{1 + \frac{(\sigma_A / S)P}{k_1 h\nu} + \frac{(\sigma_A / S)P}{k_2 h\nu} + \frac{0.9(\sigma_A / S)^2 P^2}{k_1 k_2 (h\nu)^2}} \tag{S26}$$

Because $\frac{(\sigma_A / S)P}{k_2 h\nu} \ll \frac{(\sigma_A / S)P}{k_1 h\nu} \ll 1$, Eq.S26 can be simplified as

$$\Delta P \approx \frac{\frac{0.9\sigma_A(\sigma_A / S)P}{k_2 h\nu}\cdot P / S}{\frac{(\sigma_A / S)P}{k_2 h\nu} + \frac{0.9(\sigma_A / S)^2 P^2}{k_1 k_2 (h\nu)^2}} = \frac{k_1 \cdot (0.9\sigma_A / S)\cdot P}{k_1 + (0.9\sigma_A / h\nu S)\cdot P} \tag{S27}$$

We note that single gold nanoparticle result Eq.S27 is similar to the single molecule result Eq.S4.

In our CW pump-probe experiment, when the pump beam is on, the attenuation of the probe beam by the gold nanoparticle equals

$$\Delta P_{on} = \frac{k_1 \cdot (0.9\sigma_A / S) \cdot P_{probe}}{k_1 + (0.9\sigma_A / h\nu S) \cdot (2P_{pump} + P_{probe})} \quad \text{(S28)}$$

When the pump beam is off, the attenuation of the probe beam equals

$$\Delta P_{off} = \frac{k_1 \cdot (0.9\sigma_A / S) \cdot P_{probe}}{k_1 + (0.9\sigma_A / h\nu S) \cdot P_{probe}} \quad \text{(S29)}$$

The modulation depth of the transmitted probe beam is therefore

$$\frac{\delta\delta P}{P_{probe}} = \frac{\Delta P_{off} - \Delta P_{on}}{P_{probe}} = \frac{2k_1 \cdot (0.81{\sigma_A}^2 / S^2 h\nu) \cdot P_{pump}}{[k_1 + (0.9\sigma_A / h\nu S) \cdot P_{probe}][k_1 + (0.9\sigma_A / h\nu S) \cdot (2P_{pump} + P_{probe})]}$$

Given a beam waist area of:

$$S = \pi r^2 = \pi(\frac{0.61 \times \lambda}{2 \times N.A.})^2 = \pi(\frac{0.61}{2 \times 1.4} \times \frac{(520+532) \times 10^{-7}\,\text{cm}}{2})^2 = 4.12 \times 10^{-10}(\text{cm}^2)$$

an average photon energy of:

$$h\nu = h\frac{c}{\lambda} = 6.63 \times 10^{-34} \times \frac{3 \times 10^8}{(520+530)/2 \times 10^{-9}} = 3.78 \times 10^{-19}(J)$$

absorption cross section $\sigma_A = 4 \times 10^{-12} cm^2$, pump and probe power $P_{probe} = P_{pump} = 450\mu W$, and relaxation rate $k_1 = 2.5 \times 10^{11} s^{-1}$, the signal can be calculated as

$$\frac{\delta\delta P}{P_{probe}} = \frac{2 \times 2.5 \times 10^{11} \times \frac{0.81 \times (4 \times 10^{-12})^2}{3.78 \times 10^{-19} \times (4.12 \times 10^{-10})^2} \times 450 \times 10^{-6}}{[2.5 \times 10^{11} + \frac{0.9 \times 4 \times 10^{-12} \times 450 \times 10^{-6}}{3.78 \times 10^{-19} \times 4.12 \times 10^{-10}}][2.5 \times 10^{11} + \frac{0.9 \times 4 \times 10^{-12} \times 1350 \times 10^{-6}}{3.78 \times 10^{-19} \times 4.12 \times 10^{-10}}]} = 1 \times 10^{-4}$$

Figure 2c reveals the ground state depletion signal for a single 20nm gold nanoparticle is $\delta\delta P/P$ ~ 0.5×10^{-4}. The theoretical estimate and experimental results reach a reasonable agreement. The difference between them can be explained by the rough estimation of the fast/slow component ratio and relaxation rates that we get from time-resolved spectroscopy which was not conducted under exactly the same experimental conditions (excitation wavelength, solvent for the nanoparticles, etc).

Calculation of the ground state depletion signal from a single Atto647N molecule

Again, the difference in cross-section σ, S and $h\nu$ for wavelengths 633nm and 647nm is neglected. The extinction coefficients ε of Atto647N at 633nm and 642nm in aqueous solution are,

ε (633nm) =116,000 $M^{-1} \cdot cm^{-1}$
ε (642nm) =148,000 $M^{-1} \cdot cm^{-1}$

According to the relation

$$\sigma = 3.82 \times 10^{-21} \varepsilon \quad \text{(S30)}$$

where σ is in the unit of cm^2 and ε is in the unit of $M^{-1} \cdot cm^{-1}$, the average absorption cross-section in the spectral region between 633nm and 647nm equals to

$$\sigma = 3.82 \times 10^{-21} \times \frac{(1.16+1.48) \times 10^5}{2} = 5.04 \times 10^{-16}(\text{cm}^2)$$

The beam waist area S for a 1.4 N.A. objective, 633nm and 642nm wavelengths,

$$S = \pi r^2 = \pi(\frac{0.61\times\lambda}{2\times N.A.})^2 = \pi(\frac{0.61}{2\times 1.4}\times\frac{(633+642)\times10^{-7}\,\mathrm{cm}}{2})^2 = 6.06\times10^{-10}(\mathrm{cm}^2)$$

The average photon energy,

$$h\nu = h\frac{c}{\lambda} = 6.63\times10^{-34}\times\frac{3\times10^8}{(633+642)/2\times10^{-9}} = 3.12\times10^{-19}(J)$$

The relaxation rate of Atto647N,

$$k_{relax} = \tau^{-1} = \frac{1}{3.4\times10^{-9}} = 2.9\times10^8(s^{-1})$$

where τ is the excited state lifetime of Atto647N, which is 3.4 ns.[S4]

The power levels of pump and probe beams,

$$P_{pump} = 350\ \mu\mathrm{W}$$
$$P_{probe} = 350\ \mu\mathrm{W}$$

Substituting σ, S, $h\nu$, k_{relax}, P_{pump}, and P_{probe} into Eq.S8, the modulation depth transferred by a single molecule,

$$\frac{\delta\delta P}{P} = \frac{2\times2.9\times10^8\times(\frac{5.04\times10^{-16}}{6.06\times10^{-10}})^2\times\frac{350\times10^{-6}}{3.12\times10^{-19}}}{\left(2.9\times10^8+\frac{5.04\times10^{-16}\times350\times10^{-6}}{6.06\times10^{-10}\times3.12\times10^{-19}}\right)\times\left(2.9\times10^8+\frac{5.04\times10^{-16}\times(2\times350+350)\times10^{-6}}{6.06\times10^{-10}\times3.12\times10^{-19}}\right)} = 1.2\times10^{-7}$$

Calculation of shot noise during the scanning experiment for single Atto647N molecules

Assuming 70% collection efficiency, the number of photons that arrive the photodiode during 30 ms integration time for 350 μW incident power at 633 nm,

$$\#\ \text{photon per 30 ms} = \frac{P\cdot t\cdot0.7}{h\frac{c}{\lambda}} = \frac{350\times10^{-6}\times0.7\times30\times10^{-3}}{6.63\times10^{-34}\times\frac{3\times10^8}{633\times10^{-9}}} = 2.34\times10^{13}.$$

Therefore the instant shot noise,

$$\text{shot noise for 30 ms integration} = \frac{1}{\sqrt{2.34\times10^{13}}} = 2.1\times10^{-7}.$$

The instant shot noise can be reduced by averaging the lines that scanned across a molecule. Molecule A in Figure 4a survives 45 line scans, the shot noise is reduced to

$$\frac{2.1\times10^{-7}}{\sqrt{45}} = 3.1\times10^{-8}.$$

Molecule B in Figure 4c survives 44 line scans, the shot noise is reduced to

$$\frac{2.1\times10^{-7}}{\sqrt{44}} = 3.2\times10^{-8}.$$

Modulation frequency dependence of ground state depletion signals

Using the ground state depletion microscope, we imaged single 20 nm gold nanoparticles in a same field of view with modulation frequencies in the range from 750 kHz to 7 MHz. The power level at laser foci for each beam is 450 μW. Figure S2 shows that the signal intensity is independent of modulation frequencies.

Using the ground state depletion microscope, we imaged aggregates of Atto647N in the PMMA matrix with modulation frequencies in the range from 60 kHz to 8 MHz. The power level at laser foci for each beam is 350 μW. We prepared a homogeneous layer of Atto647N molecules by spin-coating a 200 nanomolar solution of Atto647N (diluted into a poly(methyl methacrylate) matrix, 3% mass PMMA, Polysciences, MW 75,000) onto cleaned No.1 microscope cover slips. A thin layer of polyvinyl alcohol is coated on top of the PMMA layer. To avoid the complication by photobleaching of Atto647N, we changed the field of view before imaging at a new modulation frequency. Figure S3 shows that the signal intensity is independent of modulation frequencies.

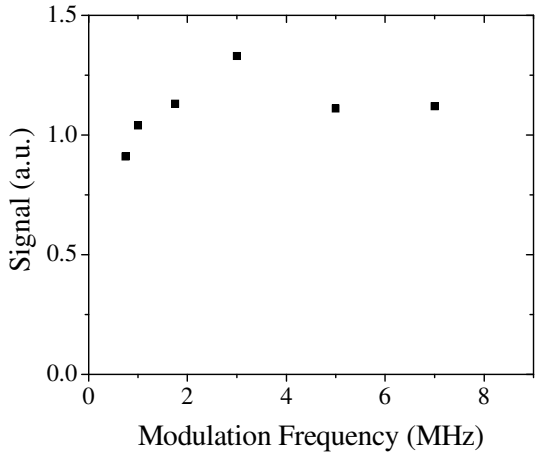

Figure S2. Ground state depletion signal of 20 nm gold nanoparticles measured with different modulation frequencies.

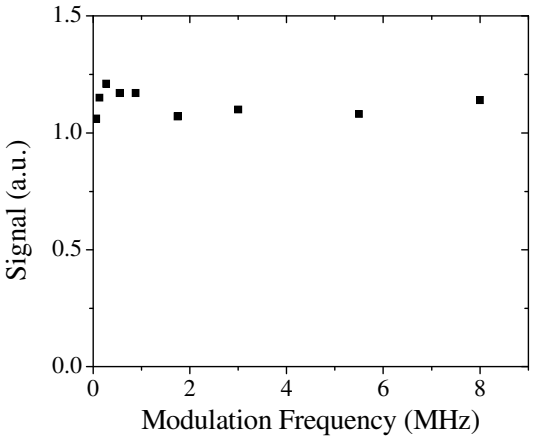

Figure S3. Ground state depletion signal of Atto647N aggregates in PMMA matrix measured with different modulation frequencies.

Single-molecule survival times and detectability of single-molecule ground state depletion signals

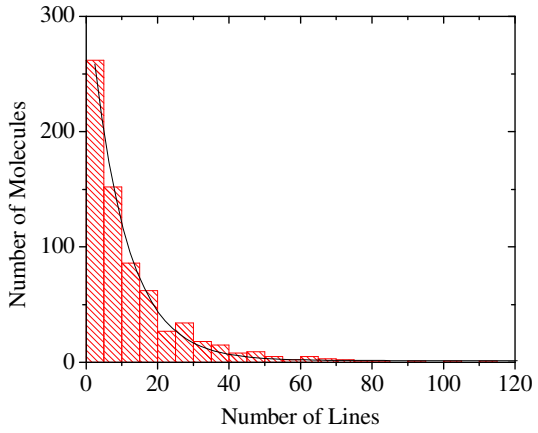

Figure S4. Occurrence of number of line scans before photobleaching for 690 single Atto647N molecules.

Figure S4 shows the number of line scans before single Atto647N molecules photobleach during simultaneous acquisition of fluorescence and ground state depletion signals. The histogram can be fitted with an exponential decay (black curve). A single Atto647N molecule survives 13 lines on average under our experimental condition. In principle, averaging about 20 line scans are required to reduce noise to allow ground state depletion signal from a single molecule to be detected. For the 690 molecules as shown in Figure S4, 19% of them survive longer than 20 lines. We successfully detected ground state depletion signal from 54% of these long-lived molecules. The fact that we didn't detect all of the long-lived molecules can be explained by the random orientation of absorption dipole moments and the inhomogeneity of the single-molecule absorption spectrum.

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